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L -Carnitine and Melatonin Reverse CCl_4 Induced Liver Fibrosis In Rats (Histological and Histochemical Studies)

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Abstract

Carbontetrachloride (CCl_4) is closely related chemically to chloroform and likewise in hepatic poisons. This study was designed to evaluate the effects of carbon tetrachloride on liver of male rats and the reversing effects of L-carnitine and melatonin on established liver fibrosis. A total of 72 adult male albino rats were used in this study. The animals were divided into six groups. Group (1) animals of the first group were kept as control andtreated with paraffin oil twice weekly for eight weeks. Group (2) rats of the second group were injected with CCl_4 intraperitoneally at 0.15 ml per rats (diluted 1:1 in liquid paraffin) twice weekly for eight weeks to produced liver fibrosis. Group (3) following establishment with CCl_4 which induced liver fibrosis, the rats were treated with L-carnitine at a dose level of 50 mg/kg for four weeks. Group (4) rats with liver fibrosis were injected intraperitoneally with melatonin at dose level of 10 mg/kg for four weeks. The fifth and sixth groups were given L-carnitine and/or melatonin at dose levels of 50 mg/kg and 10 mg/kg respectively for four weeks.

Histological changes in the liver of rats treated with CCl_4 including liver fibrosis, architecture distortion and appearance of many pseudolobule. The fibrous tissues run in septa between the nodules. The liver damage varied from one area to another and varied from moderate fibrosis to cirrhosis.

Quantitative measurement of the severity of liver fibrosis (area damage) was achieved by using computerized image analysis (Leica image) showed that highly significant increase in area of fibrosis was recorded in the case of rats treated with CCl_4 only.

Quantitative DNA image analysis showed that 3% of aneuploid cells could be noticed in liver of rats treated with CCl_4 only. Histochemical results of rats treated with CCl_4 showed highly significant increase in grey level of mucopolysaccharides and protein levels. No histological and histochemical changes could be noticed in the liver of rats treated with either L-carnitine or melatonin only. Both L–carnitine and melatonin were found to reverse CCl_4 induced liver damage.

Keywords: L-carnitine, melatonin, carbontetrachloride, liver fibrosis, rats, histological, histochemical.

Introduction

Carbontetrachloride (CCl_4) are group of hydrocarbons. All hydrocarbons, are central nervous system (CNS) depressant. Aromatic and halogenated hydrocarbons are rapidly absorbed and distributed into the central nervous system, liver and kidney. It is closely related chemically to chloroform and likewise in hepatic poisons (Wilson, *et al*., 1991). Carbontetrachloride has a wide spread use in various industries as a solvent (El-Dessoky, *et al.*, 1978). CCl_4 has been introduced into home as fire extinguisher and dry cleaner under the trade name of "pyrene" and "carbona". CCl_4 is sometimes used by hair dressers as dry shampoo (Rubbin, et al., 1984). Chronic exposure to CCl_4 lead to plastic anemia and irreversible bone marrow damage (Sullivan and Krieger, 1992).

L – carnitine is synthesized in the body from the amino acids lysine and methionine. L–carnitine is a valuable as a high quality supplement from body building come, as well as from natural and synthetic

sources (Brass, *et al*., 2001). L–carnitine is a very similar to the non-essential amino acid carnitine. It performs some of the same functions such as helping metabolized food into energy (Barker, *et al.*, 2001). It is used for fat burning increasing energy, improving resistance to muscle fatigue (Hiatt, *et al*., 2001) and very important in patients with diabetes and high blood pressure (Digiesi *et al.*, 1989). It is very important in infancy and in situation of high energy needs, such as pregnancy and breast feeding women (Giovannini *et al.*, 1991). L – carnitine has been given to people with chronic lung disease (Dal-Negro *et al*., 1988), it is useful in increasing the heart output and improving its functioning as well as stimulating the heart energy supply and improving cardiac performance (Colonna and Iliceto, 2000). Deficiency of L – carnitine is occasionally associated with some diseases such as diabetes and cirrhosis (Kender, 1986). In Italy, L–carnitine is prescribed for heart failure, heart arrhythmias, angina, and lack of oxygen to the heart (Delfavero, 1988). L–carnitine significantly decreased triglyceride, cholesterol and phospholipid in rat fed olive oil (Maccari *et al.*, 1987). It may prevent lipid peroxidation and thus may protect against liver damage (Demirdag *et al*., 2004).

Melatonin is a hormone produced especially at night in the pineal gland. The secretion is stimulated by the dark and inhibited by light (Wurtman *et al*., 1995). Melatonin has been found to be the most effective scavenger of highly toxic free radical which induced DNA damage (Tahan *et al*., 2004). Melatonin has been found to be efficacious in delayed sleep phase syndrome (Oldani *et al*., 1994), and antagonizes the mitogenic effects of estrogen (Tahan *et al*., 2004). Melatonin may augment the antitumer activity of IL-2 (interleukin 2) by inhibiting tumour growth factor production (Webb and Pvig-Domingo, 1995), it may be beneficial in the treatment of postmeno-pousal osteoporosis and it is very important in regulation of calcium and phosphorous metabolism by stimulating the thyroid glands and by inhibition calcitonin release and postglandin synthesis in animals. Melatonin helps to decrease hydroxyl radical concentration in the postischemic reprefused heart (Reiter *et al*., 1995).

Recent studies have reported that melatonin is a potent free radical scavenger, on mercury induced kidney failure (Sener *et al*., 2004), it is very important as neuroprotective role and protect erythrocytes from impaired deformability in sodium nitroprusside (SNP) (Aydogan *et al*., 2004). It may prove to be useful therapeutic agent in the treatment of age related congnitive decline (Peck *et al.*, 2004), and it is significantly suppressed the nitric oxide (NO) induced in mean inner retinal thickness (Siu *et al*., 2004). Melatonin has been introduced as an oncostatic agent especially for hormone dependent tumor (Suzme *et al.*, 2004) and provide protection against cyclophosphamide induced tissue damage (Manda and Bhatia, 2003), nephrotoxicity induced by adramycin (Tunez *et al*., 2003).

The aim of the present study is to investigate the reversing effects of L-carnitine and melatonin against CCl_4 induced chronic liver damage in rats.

Material And Methods

Seventy two – (2 months old) male albino rats were purchased from the Animal House of National Research Center. The rats were divided into seven groups , 12 rats eacn. Group (1) animals of the first group were kept as control and treated with paraffin oil twice weekly for eight weeks. Group (2) 36 rats of the second group were injected with CCl_4 intarperitoneally at a dose level 0.15 ml per rat (diluted 1:1 in liquid paraffin) twice weekly for eight weeks to produced liver fibrosis, then 12 rats were killed and liver was taken. Following establishment of CCl_4 induced liver fibrosis, 12 rats were taken and given L- carnitine (50 mg/kg) by gavage at total period for four weeks Group (3). The remaining rats with liver fibrosis were injected intraperitoneally with melatonin at a dose level of 10 mg/kg for four weeks (group 4). Group (5) animals given L-carnitine only by stomach tube at a dose

level of 50 mg/kg daily for four weeks. Group (6) animals given melatonin only at a dose level of 10 mg/kg daily for four weeks.

Histological and Histochemical studies:

The livers of different groups were removed and fixed in 10% of formol saline, 5 µm thick paraffin sections were stained with haematoxylin and eosin (Drury and Wallington, 1980) and investigated by light microscope.

Quantitative measurement:

Quantitative measurement of the severity of liver fibrosis, DNA, protein and mucopolysaccharides were achieved by using computerized image analyzer (Leica Qwin 500 image) in Image Analyzer Unit, Pathology Department, National Research Center. To measure the severity of liver fibrosis, Masson`s trichrome stained sections were used (Masson, 1929). The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Ten fields were chosen in each specimen and the mean values were obtained.

To measure the grey levels of the mucopolysaccharides and the total proteins in slides stained with special stains (Mac-Manus & Cason, (1950) and Mazia, *et. al.* (1953) respectively), the areas of reactivity were masked and the optical density of mucopolysaccharides and total proteins reaction was measured in the cytoplasm of hepatocytes using the grey image menu in ten small measuring frames in each specimen. The image was transformed into a grey image [a grid of pixels each representing the intensity or brightness at that point by a range of numbers, typically from 0 (black) to 255 (white)]. This means, when the readings are nearest to the black the highest contents of total protein or mucopolysaccharides are found and when the readings are nearest to the white the small amount of the protein or mucopolysaccharides are found.

DNA content analysis was performed on sections stained by Feulgen methods (Feulgen and Rosenback, 1942). For each section 100-120 cells were randomly measured. The threshold values are defined by measuring control cells. The result is presented as a histogram, in which normal diploid cells are separated clearly from aneuploidy cells.

Statistical analysis

All statistical analysis calculation involved as analysis of variance (ANOVA) and the student t-test. Descriptive statistics are given as mean ± SE. Analysis of variance (one-way ANOVA) was done, with student t-test. Differences were considered signify-cant when $p<0.05$.

Results

Histological results :

The liver of control rats revealed the normal characteristic hepatic architecture (Fig. 1).

No pathological changes could be noticed in the liver of rats treated with each of L- carnitine and melanoma.

Chronic treatment of rats with CCl_4 for 8 weeks showed cirrhotic nodules and marked fibrosis. The degree of cirrhosis varied from moderate to severe (Fig. 2).

The microscopic examination in cirrhotic nodules showed marked fibrosis, architecture distortion and appearance of many pseudolobules without no relationship to normal hepatic vascularity. The fibrous tissues run in septa between the nodules. The liver tissues showed well formed regenerative nodules composed of displastic cells (Fig. 3). A large haemorrhagic area could be observed (Fig. 4).

The liver of rats treated with L-carnitine and CCl_4 (group 3) showed some protective effects as compared to group of rats subjected to CCl_4 only in the form of diminution of cirrhosis. While moderate fibrosis, vacuolar degeneration and fatty changes were also seen. Large and small haemorrhagic areas could be noticed (Fig. 5).

Concerning rats treated with melatonin, then subjected to CCl_4 (group 4), examination of liver sections showed macro

and micro-vacuolar fatty degeneration, and signs of nuclei degeneration in hepatocytes in the form of some pyknosis, karyolysis and karyorrhexis. (Fig. 6)

Image analysis of liver fibrosis:-

Examination of control liver sections showed collagen occurred as wavey fibril, either singly or fused together in dense bundles (Fig. 7 a).

Liver fibrosis was assessed directly by hepatic morphometric analysis, which has been considered as the gold standard for quantification of fibrosis.

Significant increase in the area of fibrosis ($P < 0.05$) was observed in the group of rats treated with CCl_4 only (Table 1 & Fig. 7 b, c) as compared to control. Following establishment of liver fibrosis the rats treated with each of L–carnitine and melatonin showed significant decrease ($P < 0.05$) in area of fibrosis as compared to rats treated with CCl_4 only (Table 1 & fig. 7 d & e)

Histochemical results

DNA content in all studied group:-

Image analyzer automatically express the DNA content of each individual cell measured then gave the percentage of each cell out of the total number of cells examined and classified the cells into four groups namely, diploid (2c), proliferation index (S-phase cells) (3c), tetraploid (4c) and cells with more than 4c DNA content (> 4 c) which indicate aneuploidy.

Table 2 & Figs. 8, 9 a, show the DNA content in normal rats.

In the present work, the liver of rats treated with CCl_4 only showed 3 % of the examined cells more than 5c (aneuploidy), 54 % of cells contained diploid DNA value (2c), 18 % of the examined cells contained 3c DNA value (proliferation index was high), 8 % of the examined cells were at the 4 c area (tetraploidy). 15 % of the examined cells contained DNA < 1.5 c (Table 3 & Figs. 8, 9 b).

In the present work, the treatment of rats with L-carnitine and CCl_4 showed no DNA aneuploidy, 54.9 % of the examined cells contained diploid DNA value (2c), 30.3 % of cells contained 3c DNA value (proliferation index was high), 3.9 % of the cells were at 4 c area value (tetraploidy) (Table 5 & Figs. 8, 9 c).

Concerning rats treated with melatonin and CCl_4 75.7% of the examined cells contained diploid DNA value, 16.8 % of the examined cells contained DNA <1.5c, 6.5 % of the examined cells contained 3c DNA value (proliferation index was low), 0.9 % of the examined cells were at the 4 c area (tetraploidy) and no DNA aneuploidy could be noticed (Table 4 & Figs. 8, 9 d).

Examination of the control liver sections stained with periodic acid Schiff`s (PAS) showed the mucopolysaccharides content in the cytoplasm of hepatocytes; the peripheral zonal cells showed higher muco-polysaccharides content than the central zonal cells (Fig. 10 a).

The rats treated with CCl_4 only exhibited a significant decrease ($p < 0.05$) in mucopolysaccharides content (increase in grey level) of liver cells relative to control value (Table 6 & Fig. 10 b).

Significant increase ($p < 0.05$) in mucopolysaccharides content was recorded also in case of rats given CCl_4 and L-carnitine (group 4) and CCl_4 melatonin (group 3) (decrease in grey level) as compared to rats treated to CCl_4 only (Table 6 & Figs. 10 c &d).

Examination of control liver sections showed moderate protein content in the cytoplasm of hepatocytes. Some nuclei showed deep protein content (Fig. 11 a).

The liver of the rats treated with CCl_4 only exhibited a significant decrease ($p < 0.05$) in protein content in the cytoplasm of hepatocytes as compared to control (increase in grey level) (Table 7 & Fig.11 b).

A significant increase in protein content ($P< 0.05$) was recorded in the case of rats subjected to CCl_4 and L – carnitine or CCl_4 and melatonin (decrease in grey level) as compared to CCl_4 only (Table 7& Fig.11 c& d)

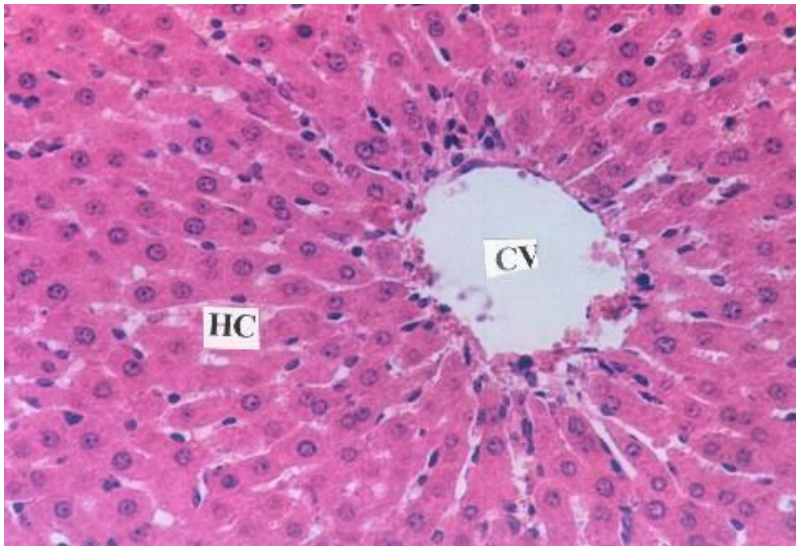


Fig. (1): Section in the liver of control rat showing normal histological structure of hepatic lobules and central vein. **(H &E X 200)**

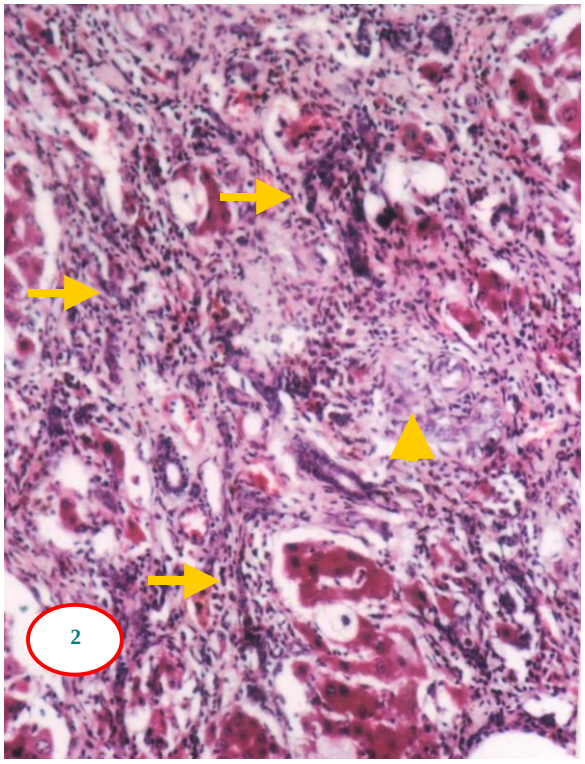


Fig. (2): Section of the liver of a rat treated with CCl_4 showing marked fibrosis (arrow) and cirrhosis (arrowhead) **(H &E X 100)**

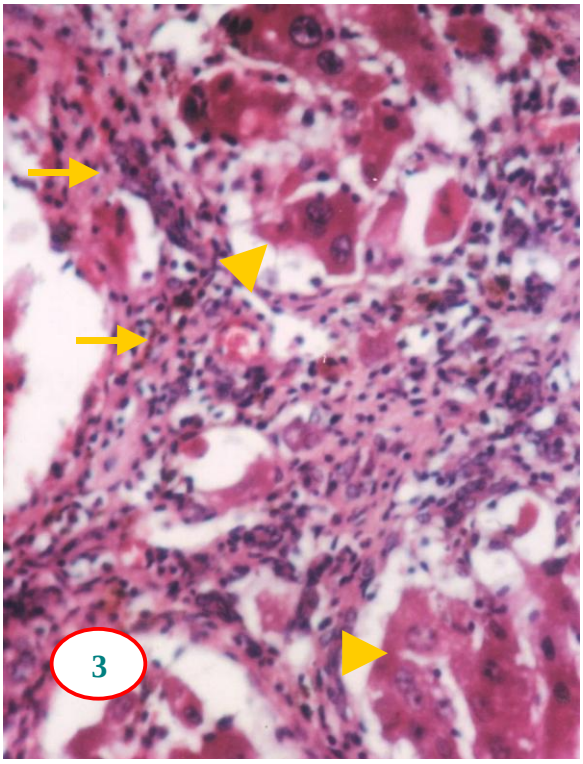


Fig. (3): Section of the liver of a rat treated with CCl_4 showing the fibrous tissues run in septa (arrow) between the nodules and displastic cells (arrowhead). Many pesudolobules could be noticed.**(H &E X 200)**

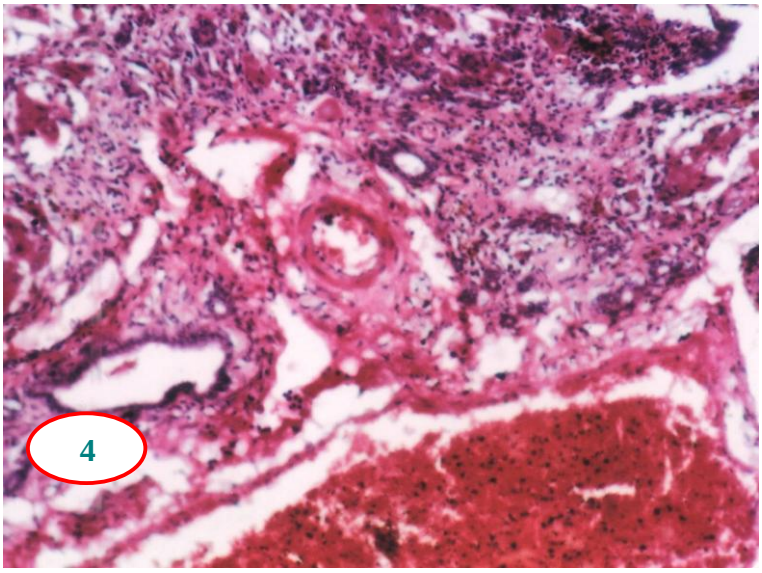


Fig. (4): Section of the liver of a rat treated with CCl_4 showing severe hemorrhage. **(H & E X 100)**

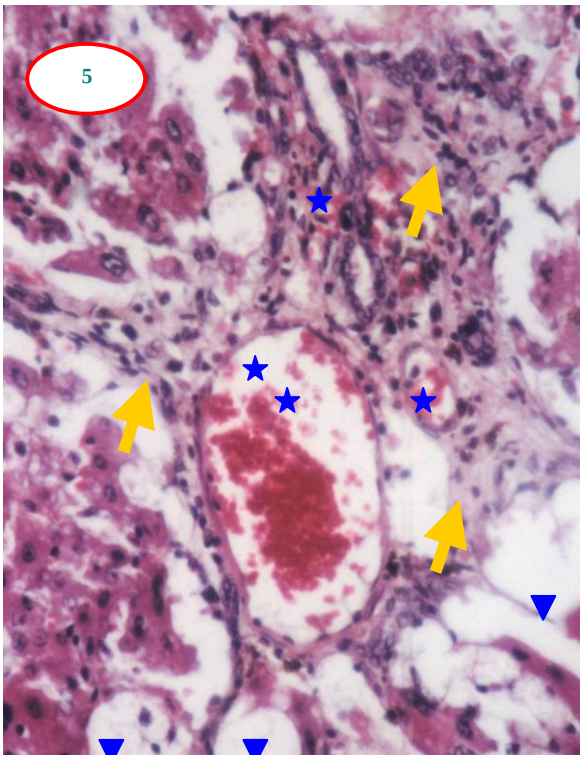


Fig. (5): Section of the liver of a rat treated with CCl_4 and L-carnitine showing moderate fibrosis (arrow), fatty changes and vacuolar degeneration (V) with large and small haemorrhagic areas (Stars). No cirrhosis **(H &E X 200)**

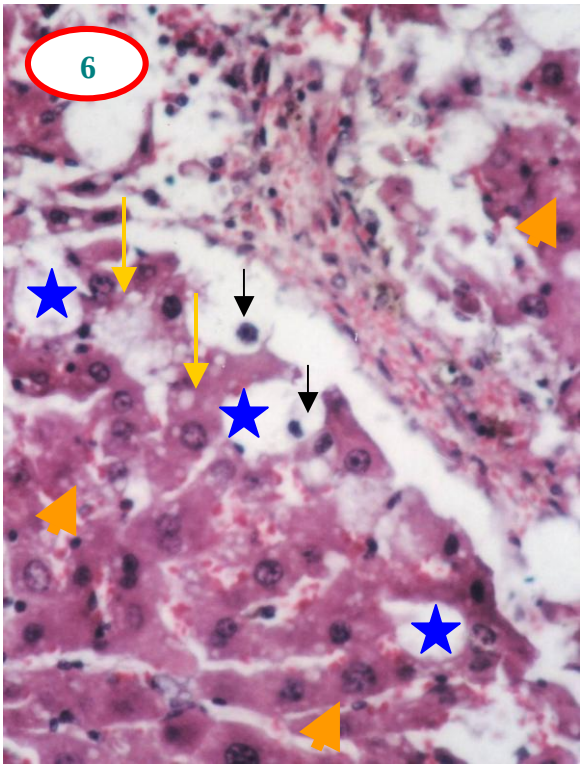
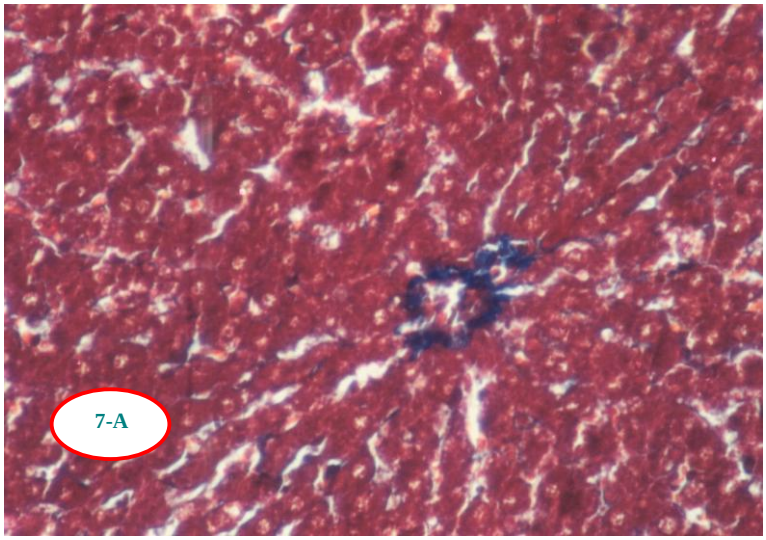
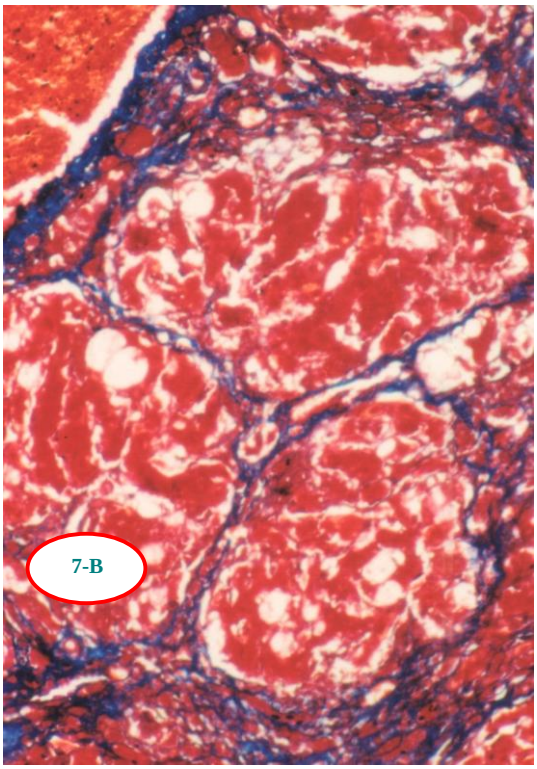


Fig. (6): Section of the liver of a rat treated with CCl_4 and melatonin showing macro (stars) and micro (yellow arrow) vacuolar degeneration, Fatty degeneration and some pyknotic (black arrow), karolysis and karyorrhexis nuclei (arrowhead). **(H &E X 200)**


7-A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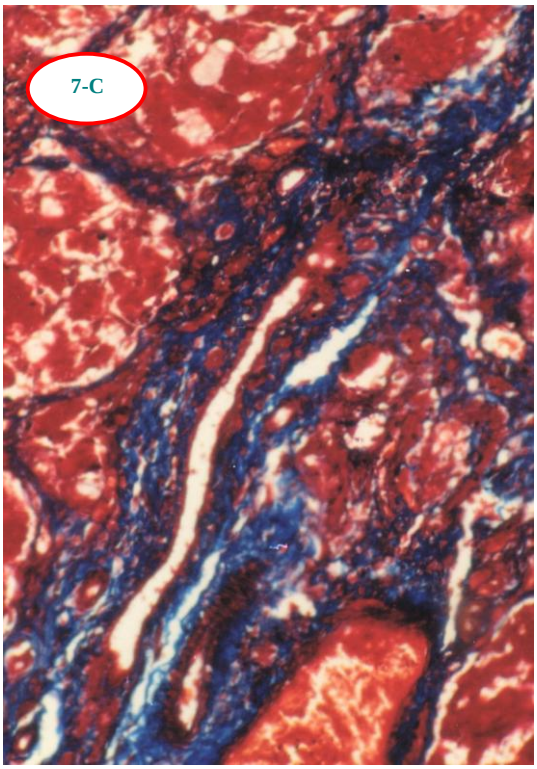
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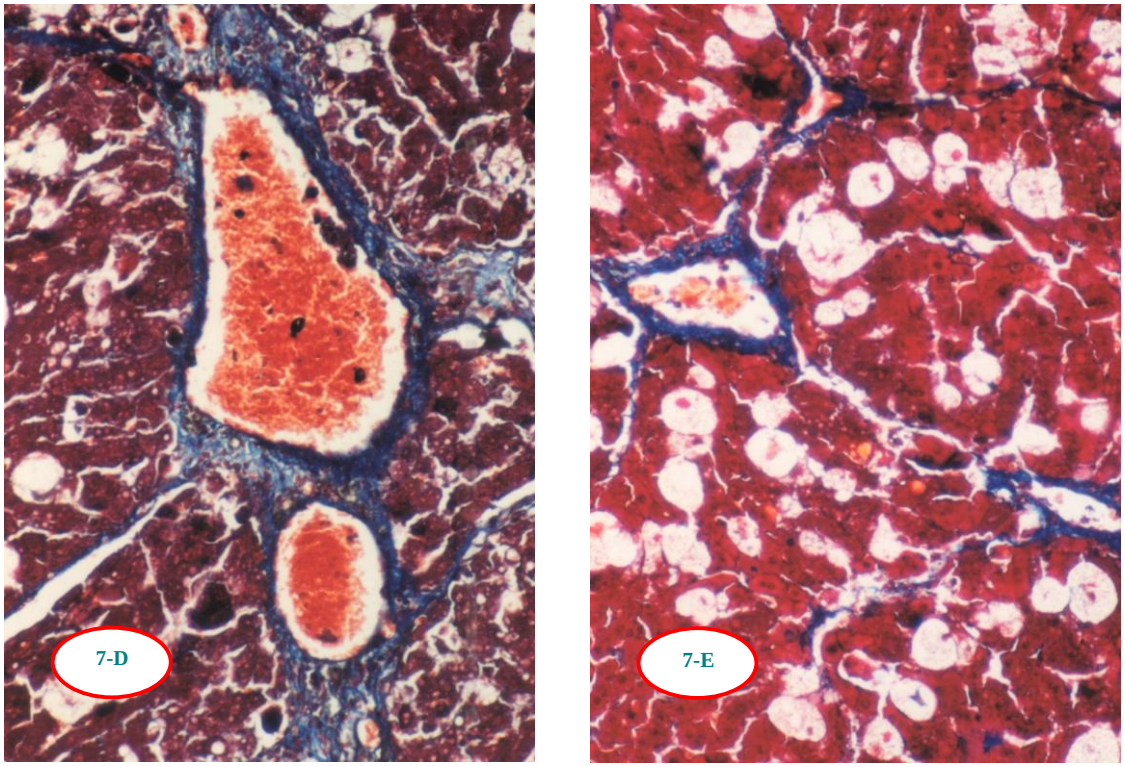


Fig. (7): Section of the liver of a rat showing collagen occurred as wavy fibrils either singly or fused together in dense bundles. **(A):** Control **(Masson trichrome stain X 100)**. **(B & C)**: Treated group with CCl_4 showing increase deposition of fibrous tissues and connective tissue. **(D):** Treated group with CCl_4 and L- carnitine showing moderate amount of fibrous tissues. **(E):** Treated group with CCl_4 and melatonin showing mild deposition of fibrous tissues.**(Masson trichrome stain X 200)**

Table (1): qualitative study of the area of fibrosis in different groups

	Area	Area Fraction	Area%
Control	0	0	0
CCl_4	57352.4± 10997.55	0.205 ± 0.039	20.524 ± 3. 947
CCl_4 & melatonin	13763.3 ± 1947.9 •	0.056 ± 0.008	5.567 ± 0.799
CCl_4 & L- carnitine	22058.8 ± 3053.154 •	0.077± 0.011	7.739 ± 1.074

At the $P < 0.05$ level, the means are significantly different when compared with CCl_4 treated group.

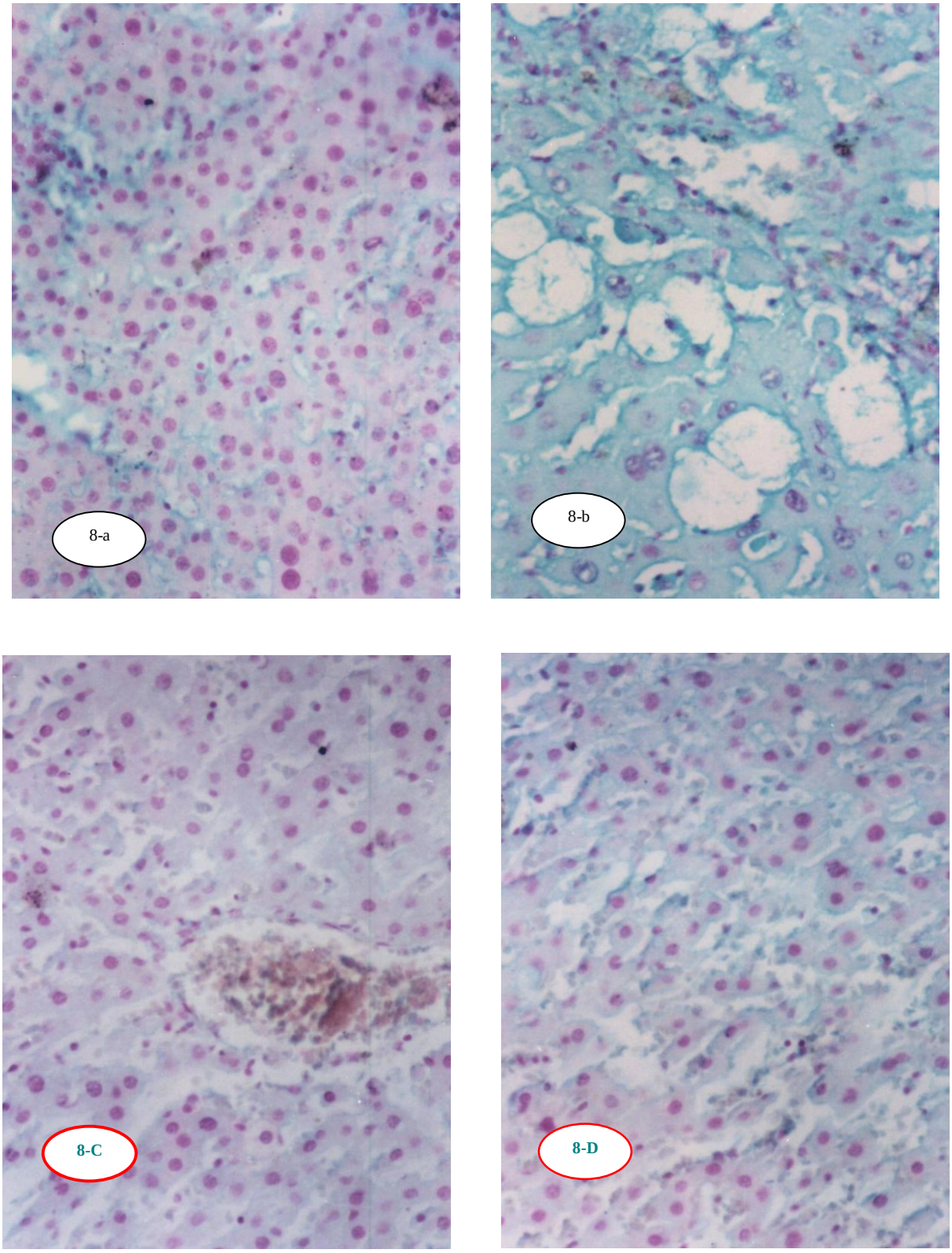


Fig. (8): Section of the liver of rat showing DNA in hepatocytes. **(A):** Control. **(B):** Treated group with CCl_4 showing a decrease in DNA content. **(C):** Treated group with CCl_4 and L- carnitine showing mild improvement in DNA content. **(D):** Treated group with CCl_4 and melatonin showing DNA content more or less approximated control.**(Feulgen reaction X 400)**

Fig. (9-a): Showing normal DNA content in hepatocytes of rats.

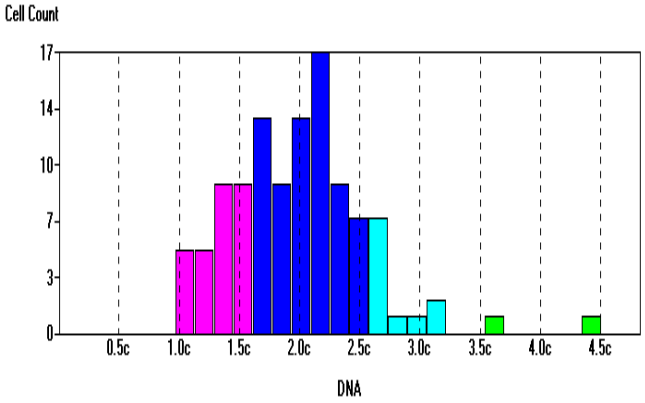


Table (2): Normal DNA content in hepatocytes of rats.

Range	Tot Cells	% Cells	DNA Index	Mean c	Mode c	Std. Dev. c	CV c	Min c	Max c
All	109	100.0	1.000	1.990	2.199	0.556	27.925	1.008	4.478
5cER	0	0.0	-	-	-	-	-	-	-
< 1.5c	22	20.183	0.646	1.286	1.363	0.152	11.846	1.008	1.499
1.5c-2.5c	71	65.138	1.007	2.055	2.014	0.267	13.317	1.501	2.469
2.5c-3.5c	14	12.844	1.372	2.731	2.656	0.212	7.729	2.512	3.210
3.5c-4.5c	2	1.835	2.023	4.026	4.478	0.639	15.860	3.575	4.478
> 4.5c	0	0.0	-	-	-	-	-	-	-

Fig. (9 -b): DNA content in hepatocytes of rats treated with CCl_4.

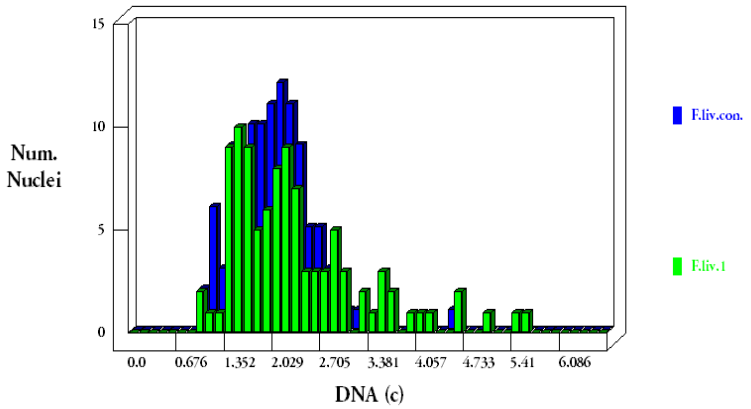


Table (3): DNA content in hepatocytes of rats treated with CCl_4

Range	Tot Cells	% Cells	DNA Index	Mean	Mode	Std. dev.	CV	Min	Max
All	100	100.0	1.182	2.352	1.434	0.956	40.654	0.949	5.612
5cER	3	3.0	2.696	5.365	5.522	0.287	5.353	5.050	5.612
< 1.5c	15	15.0	0.666	1.325	1.375	0.157	11.872	0.949	1.498
1.5c-2.5c	54	54.0	0.995	1.981	2.150	0.294	14.833	1.508	2.497
2.5c-3.5c	18	18.0	1.463	2.912	2.930	0.255	8.768	2.532	3.483
3.5c-4.5c	8	8.0	1.920	3.821	3.604	0.264	6.916	3.541	4.233
> 4.5c	5	5.0	2.549	5.072	5.024	0.449	8.848	4.615	5.612

Fig. (9-c): DNA content in hepatocytes of rats treated with L-carnitine and CCl_4.

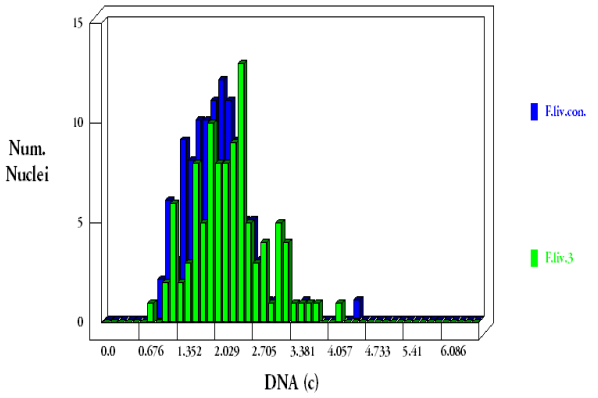


Table (4): DNA content in hepatocytes of rats treated with L-carnitine and CCl_4.

Range	Tot Cells	% Cells	DNA Index	Mean	Mode	Std. Dev.	CV	Min	Max
All	102	100.0	1.150	2.290	2.013	0.652	28.476	0.904	4.290
5cER	0	0.0	-	-	-	-	-	-	-
< 1.5c	11	10.784	0.634	1.262	1.309	0.160	12.672	0.904	1.476
1.5c-2.5c	56	54.902	1.033	2.055	2.013	0.283	13.780	1.504	2.486
2.5c-3.5c	31	30.392	1.445	2.875	2.548	0.308	10.722	2.512	3.389
3.5c-4.5c	4	3.922	1.937	3.856	3.922	0.317	8.234	3.553	4.290
> 4.5c	0	0.0	-	-	-	-	-	-	-

Fig. (9-d): DNA content in hepatocytes of rats treated with melatonin and CCl_4.

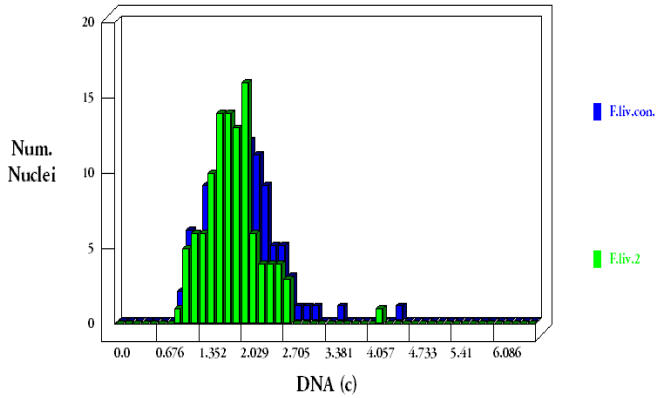


Table (5): DNA content in hepatocytes of rats treated with melatonin and CCl_4.

Range	Tot Cells	% Cells	DNA Index	Mean	Mode	Std. Dev.	CV	Min	Max
All	107	100.0	0.951	1.893	1.782	0.457	24.111	1.073	4.251
5cER	0	0.0	-	-	-	-	-	-	-
< 1.5c	18	16.822	0.645	1.284	1.344	0.116	8.999	1.073	1.442
1.5c-2.5c	81	75.701	0.970	1.931	1.751	0.251	13.003	1.501	2.481
2.5c-3.5c	7	6.542	1.351	2.689	2.743	0.080	2.961	2.589	2.789
3.5c-4.5c	1	0.935	2.136	4.251	3.420	-	-	4.251	4.251
> 4.5c	0	0.0	-	-	-	-	-	-	-

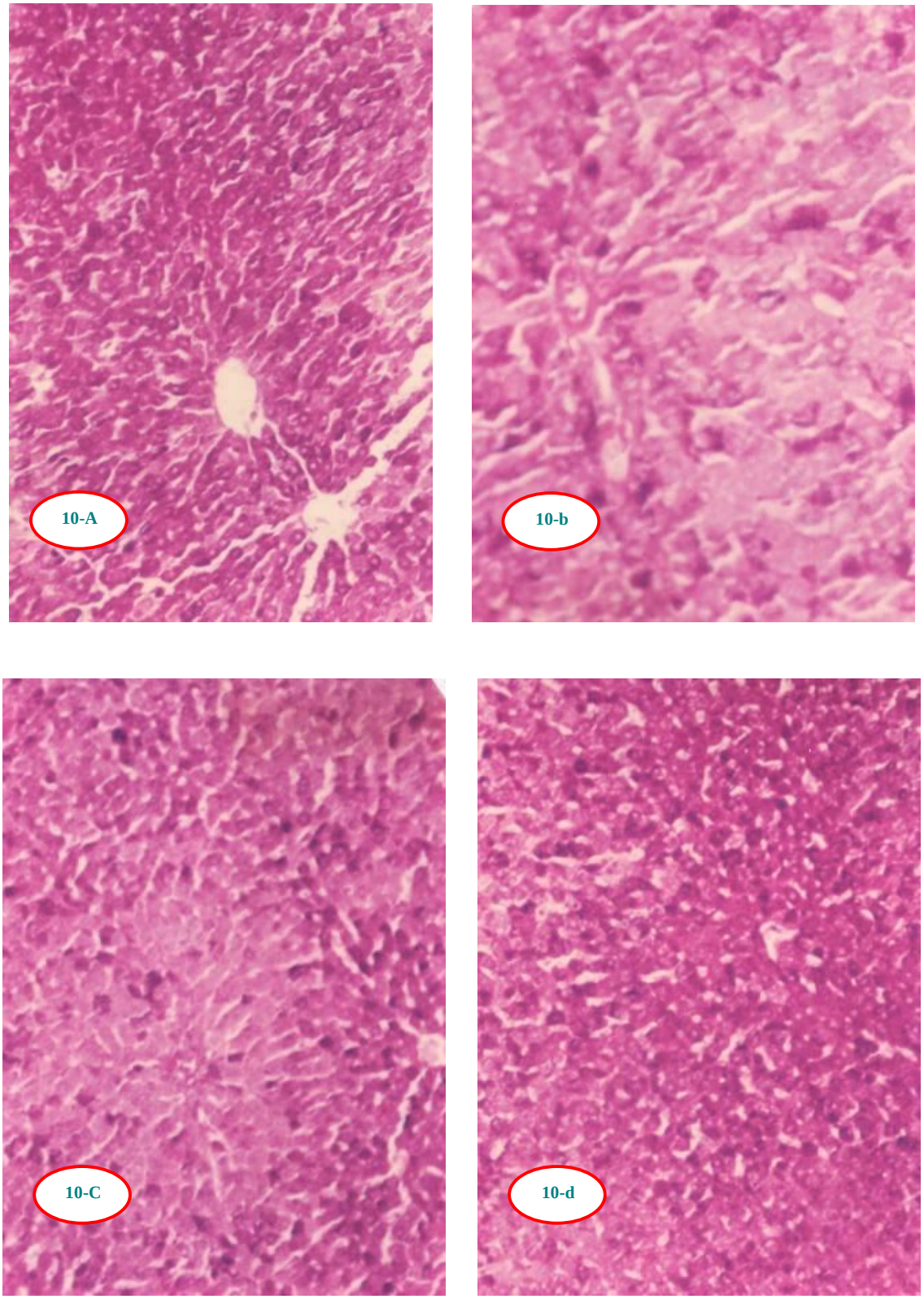


Fig. (10): Section of the liver of a rat showing mucopolysaccharides materials in the cytoplasm of hepatocytes **(A):** Control, **(B):** Treated group with CCl_4 showing marked decrease in mucopolysaccharides materials, **(C):** Treated group with CCl_4 and L- carnitine showing mild increase in mucopolysaccharides materials **(D):** Treated group with CCl_4 and melatonin showing mucopolysaccharides content more or less approximated to control. **(PAS reaction X 400)**

Table (6): Grey level of the mucopolysaccharides content of different groups.

Treatments parameters	Grey level of mucopolysaccharides Mean ± SE
Control	127.1460 ± 2.4645
CCl_4	180.7940 ± 4.5135 *
CCl_4 & melatonin	142.5640 ± 1.2871•
CCl_4 & L- carnitine	158.7630 ± 2.8479•

* At the P < 0.05 level, the means are significantly different when compared with control.

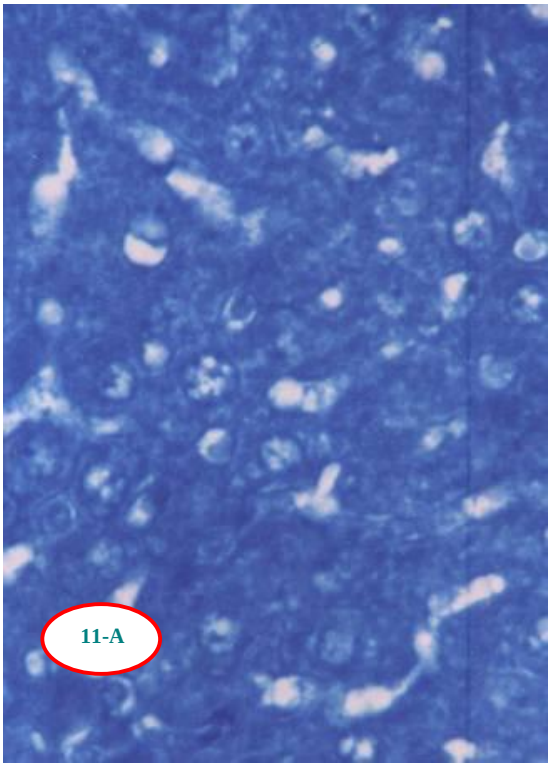
• At the P < 0.05 level, the means are significantly different when compared with CCl_4 treated group.

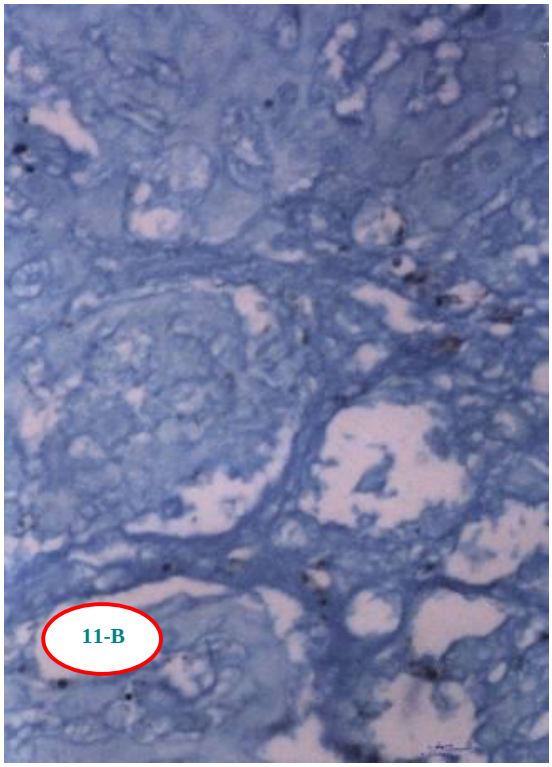
Table (7): Grey level of the total proteins content in liver of different groups .

Groups	Grey level of protein content Mean ± SE
Control	126.9820 ± 3.0627
CCl_4	210.6800 ±1.3648 *
CCl_4 & melatonin	116.6610 ± .8380 •
CCl_4 & L- carnitine	125.9800 ± 1.9325 •

*At the p < 0.05 level, the means are significantly different.

• At the P < 0.05 level, the means are significantly different when compared with CCl_4 treated group





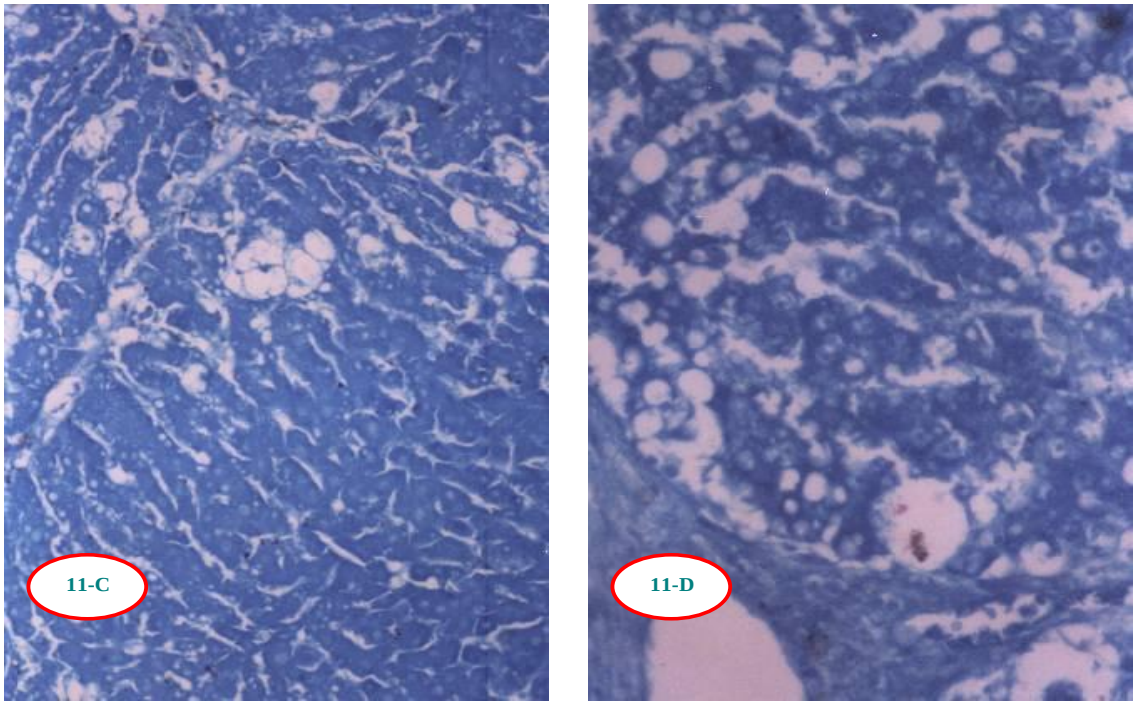


Fig. (11): Section of the liver of rat showing greenish blue protein content in the cytoplasm of hepatocytes. **(A):** Control. **(B):** Treated group with CCl_4 showing decrease of protein content. **(C):** Treated group with CCl_4 and L- carnitine showing mild increase in protein content. **(D):** Treated group with CCl_4 and melatonin showing moderate increase in protein content. **(Bromophenol blue stain X 400)**

Discussion

CCl_4 is a well known hepatotoxic chemical. The main cause of liver injury by CCl_4 is free radicals of its metabolites. Cleavage of the CCl_3 –Cl bond by superoxide (O_2) probably proceeds via the microsomal cytochrome P-450 reductase NADPH-dependent reductive pathway (Nakade *et al*., 2002). Formation of free radicals may cause lipid peroxidation and subsequent membrane injury (Propper, 1988). A decrease in hepatic blood flow is suggested as one of the important factors in aggravation of experimental liver injury induced by stimulation of hepatic sympathetic nerve (Iwai and Shimazu, 1988).

In the present work chronic administration of CCl_4 for 8 weeks induced marked fibrosis, architecture distortion and appearance of many pseudolobules. The fibrous tissues run as septa between the nodules. Results of this work were in agreement with Luo *et al.* (2004), they reported that the treatment of rats with CCl_4 for 8 weeks induced liver fibrosis. The liver exhibited a marked increase in content of extracellular matrix (ECM) and displayed bundles of collagen surrounding the lobules, which resulted in a large fibrous septae and distorted tissue architecture. The liver damage varied from one area to another and ranged from moderate fibrosis to cirrhosis. Also Huang *et al.* (2001) and Wu *et al.* (2003) reported that hepatic fibrosis was induced in rats by subcutaneous injection of 40% CCl_4 in oil solution at total period of 7 weeks. According to Neves *et al*. (2003) the treatment of rats with CCl_4 at a total period of 16 weeks caused hepatic fibrosis. The hepatic fibrosis was classified as perivenular fibrosis complete and incomplete septa fibrosis and cirrhosis. However Lin *et al*. (1999) & Germano *et al*. (2001) and Al Gamdi, (2003) they reported that the treatment of rats with CCl_4 induced hepatic lesions including fatty changes, ballooning

degeneration, cell necrosis and centrilobular inflammatory infiltrate. These finding coinciding with Turkdogan *et al*. (2003) stated that in CCl_4 treated group, necrosis and hydropic degeneration were marked in periacinar regions associated with fibrosis in the periacinar regions and in the portal tract. Hepatic fibrosis induced by CCl_4 has been explained by Wei *et al*. (2000) and Shen *et al*. (2003), they posulated that the liver fibrosis is a consequence of chronic liver injury from different causes, including alcohol, toxins, chronic viral infection and metabolic disease. In liver there is an increase deposition of extracellular matrix (ECM) in perisinusoidal and periportal spaces. The activated hepatic stellate cells (HSCs), have now been identified as the primary source of ECM in liver fibrogenesis. The pathological significance of HSCs relies on their ability to be activated into myofibroblast like cells with enhanced production of ECM. The hepatic fibrosis developed due to increased accumulation of malondialdehyde (MDA), an end product of lipid peroxidation. MDA, lipid peroxide produced in this oxidative stress cause various diseases (Lee *et al*., 2004). Chronic liver disease, such as fibrosis and cirrhosis are common in men than in women. The gender difference may be related to the effect of sex hormone on the liver (Xu *et al*., 2002). On the other hand Onell *et al*., (2000) found that histological examination of liver from CCl_4 treated rats revealed hepatocytes denatu-ration, necrosis was not obvious and pseudolobules were not found.

In the present study the treatment of rats with CCl_4 for 8 weeks showed liver with cirrhotic nodules. The degree of cirrhosis varied from moderate to sever. Results of this work were in agreement with Gonzaliez - Reimers *et al*. (2003), who reported that the treatment of rats with CCl_4 caused micronodular cirrhosis and developed in animals after 5-7 weeks of treatment. The liver cirrhosis varied from moderate to sever.

In the present work the treatment of rats with L-carnitine following establishhment of CCl_4 showed some improvement in hepatocytes compared with group of rats treated with CCl_4 only. L-carnitine prevented lipid accumulation caused by CCl_4 (Sachan and Dodson, 1992 and Sachan *et al.*, 1993). According to Demirdag *et al*. (2004) subcutaneous inject-ion of L-carnitine at a dose level of 50 mg/kg significantly reduced steotosis, inflammation and necrosis in CCl_4 treated rats.

In the present work the oral administration of rats with melatonin following establishment of CCl_4 showed some improvement in pathological changes in comparison with group of rats subjected to CCl_4 only in the form of diminution of cirrhosis. This is in agreement with Ohta *et al.* (1999) they reported that the treatment of rats with melatonin completely prevent all changes observed in CCl_4 cirrhotic rats namely lipid peroxidation and gluathione content. Coinciding with Wang *et al*. (2001) who reported that the treatment of rats with melatonin along with CCl_4, the comparative histopathological study of liver exhibited almost normal architecture. Also Daniels *et al*. (1995) reported that the treatment of rats with melatonin in combination with CCl_4. melatonin was able to counteract lipid peroxidation and enzyme leakage induced by CCl_4. According to Ohta *et al*. (2004) the treatment of rats with CCl_4 can induce the production of reactive oxygen species (ROS) which can damage the cellular elements. Melatonin post administration at pharmacological doses prevents the disruption of hepatic reactive oxygen species (ROS) metabolism associate with superoxide reductase (SOD), Catlase (CAT), glutathione reductase (GSSG-R), glucose-6-phospatase dehydrogenase (G-6-PDH) and xanthine oxidase (XO) were determined 6 and 24 h after CCl_4 treatment.

In the present work the treatment of rats with CCl_4 only showing an increase in deposition of collagen and connective tissue fibers. This is in agreement with Xu *et al*., (2004), who reported that the subcutaneous injection of rats with CCl_4 at a dose level of 2 ml/kg twice weekly at total periods of 8 weeks induced marked increase in collagen deposition. Also Favari and Pereza-Alvarez (1997), Pablo *et al.* (2003) and Siller-Lopeza, *et al*. (2004) found that the collagen content in the liver of animals

treated with CCl_4 was increased a bout four times as compared to control. The histological examination showed collagen increase distorted the normal liver architecture. According to Germano *et al*., (2001), CCl_4 caused deposition of collagen around the sinusoidal cell layer in liver of rats.

In this study the treatment of rats with L-carnitine along with CCl_4 showed more improvement in collagen deposition as compared to group of rats treated with CCl_4 only. These results were in agreement with Sachan *et al*., (1993) they noticed that the treatment of rats with L-carnitine and CCl_4, L-carnitine prevented all changes produced by CCl_4 and induced more improvement in collagen deposition.

In the present work the treatment of rats with melatonin along with CCl_4 showed moderate improvement in collagen deposition as compared to group of rats subjected to CCl_4 only. This results go in agreement with Tahan *et al*., (2004) who reported that melatonin produced inhibitory regulation of collagen content in tissues. It is a potent fibrosuppressant and antioxidants. According to Hva *et al*., (2000) melatonin increased quantity of neutral soluble collagen fraction and gene expression of minor type of collagen in normal tissue of rats. Also, Drobink and Dabrowski, (2000) who reported that treatment of rats with melatonin produced improvement of collagen deposition, matrix degradation occurs predominantly as a consequence of the action of family enzyme called matrix metalprotinases (MMPs).

DNA quantification techniques have been widely applied in studies of normal and neoplastic cell growth. The main objective of its use in the field of tumor pathology to supplement cytomorphological analytical work with quantitative cytochemical information (Auer *et al*., 1989) and Randcalli *et al*., (1989). The detection of abnormal DNA content in cells has been shown in most cases to be a reliable marker of malignancy, and the degree of DNA ploidy and / or proliferative activity has been related to the biological behavior of tumors (Hedley, 1989).

Concerning ploidy results, the treatment of rats with CCl_4 only showed that the liver contained 3% aneuploid cells (>5c), 54% of the examined cells contained diploid DNA value (2c) and high proliferation index. The dysplastic cells could be observed in histological results. These results were in agreement with Thomas *et al.* (1992), they noticed that the CCl_4 induced liver cells dysplasia and has been claimed to play a role in evaluation of hepatocellular carcinoma. This was supported by observation that the dysplastic liver cells showed aneuploid DNA patterns. Helal *et al*. (2003) showed that, aneuploid cell populations were seen in 8 out of 70 of chronic liver disease, 11.4% with DNA index (DI) ranging between 1.18% and 1.51 (mean 1.25). All these 8 biopsies had the morphological features of dysplasia. According to Kang *et al*. (2003) & Kang *et al.,* (2004) and Yoo *et al.* (2004) the abnormal DNA content are related to severity of dysplasia. In previous studies Markel *et al.,* (1987) and Auer *et al.* (1989) and Lin *et al.* (1990) they reported that the DNA aneuploid increases with increasing histological grade and stage of tumors. According to Chapman, (1995) ploidy analysis using image analysis provides more reliable ploidy results due to direct visualization and selection of cancer cells.,

In the present work the treatment of rats with CCl_4 and melatonin resulted in no aneuploid cells, 75.7 % of the examined cells contained diploid DNA value, 6.5 % of the examined cells contained 3c DNA value (proliferation index was low). These results were in agreement with Lei *et al.* (2004) and Undeger *et al.* (2004) they found that melatonin plays high protective role in repair of the DNA damage. It is provided a significant decrease in lipid peroxidation and DNA strand breakage. Moreover Suzme *et al*. (2004) found that the treatment of rats with melatonin only or along with toxic chemical agents stimulated cell proliferation and increase in DNA synthesis. According to El-Missiry and Abdel-Aziz, (2000) flow cytometric studies showed that melatonin not only delayed the progression of cells from G (0), G (1) phase to s-phase of the cell cycle but also reduced DNA synthesis during cell cycle.

Concerning histochemical results, the rats treated with CCl_4 showed decreased PAS+ve materials in the cytoplasm of the hepatocytes. These results go in agreement with Lupp et al., (2000); Sotelo-Felix et al., (2001) and Nofal *et al.*, (2002) they reported that the oral administration of a single dose of carbon tetrachloride (2.5 mg/kg b.w.; 1:1 v/v, liquid paraffin) showed marked decrease in glycogen content. Few patches of PAS+ve materials were seen. According to Kudryavtseva *et al.* (2001) the treatment of rats with CCl_4 showed that the glycogen content in cirrhotic hepatocytes were 1.2 time lower than control rats. The decrease in PAS+ve reaction in liver of rats after treatment with CCl_4 has been explained by De *et al.* (1996), they postulated that the stress caused by intoxication with CCl_4 lead to increase of glucose level, and subsequent production of liver epinephrine and thus increased glycogenolysis and this could account for the decrease in glycogen content in liver of rats.

In the previous study of Krahenbuhl *et al.* (1991) they found that the treatment of rats with CCl_4 showed a decrease in liver glycogen content by 64% as compared to control.

In the present work the treatment of rats with each L-carnitine and / or melatonin in combination with CCl_4 showed an increase in mucopolysaccharides content. These results were in agreement with Mazepa *et al*. (2000), who found that liver glycogen were significantly higher in melatonin treated animals. These results indicate that melatonin preserves glycogen stores in exercised rats though changes in carbohydrates and lipid utilization. Nishida *et al*. (1989) and Krahenbuhl *et al*. (1991), they noticed an increase in glycogen content in rats treated with L-carnitine.

In the present study the treatment of rats with CCl_4 showed a decrease in protein content in cytoplasm of hepatocytes. These results go in agreement with Nofal *et al.* (2002) and Hassan *et al*. (2003) they found that the oral administration of a single dose of CCl_4 caused a decrease in protein content in the cytoplasm of hepatocytes. Also Agha *et al.* (1995) found that the very important alteration produced by CCl_4 in the liver cells is the inhibition of protein synthesis by interaction with SH group. According to Sundari and Ramakrishna (1997) CCl_4 is converted to CCl_3 by P450 which is a highly toxic molecule. It diffuses through the cell membrane and caused lipid peroxidation of the membrane. Ribosome disagg\regates resulting in decreased protein synthesis lead to no formation of aplipoprotein causing accumulation of lipids and fatty changes.

In the present study, the treatment of rats with each L-carnitine and melatonin along with CCl_4 showed an increase in protein content in the cytoplasm of hepatocytes. These results go in agreement with Ohta *et al.* (2000) who found that the treatment of rats with melatonin caused increase in protein content in liver cells. According to Mayerly *et al.* (2000) melatonin is a well known antioxidant that protect DNA, protein and lipid from free radical damage. The increase in protein content indicating that melatonin is more effective in improving liver cell function induced by CCl_4.

According to Qwen *et al.* (2001) L-carnitine caused decreased lipid deposition and increased protein accretion in pig. Carnitine is more able to use fat for energy divert carbon towards synthesis of amino acid and spare branched chain amino acid for protein synthesis.

Conclusion

The treatment of rats with each of L-carnitine and /or melatonin after the establishment of CCl_4 which induced hepatic fibrosis significantly reduces and even reverses the fibrosis in rats. Moreover the L-carnitine and melatonin showed protective role in histological results as well as histochemical parameters. It was found that melatonin showed highly protective effect than L-carnitine.

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دراسة التأثير المعاكس ل لـ -كرنيتين والميلاتونين فى منع رابع كلوريد الكربون من تليف الكبد فى الجرذان (دراسة هستولوجية وهستوكيميائية)

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يرجع تأثير سمية رابع كلوريد الكربون على الكبد إلى انتمائه كيميائيا إلى مجموعة الكلوروفورم.
تهدف هذه الدراسة إلى تقييم التأثيرات الهستولوجية والهستوكيميائية الناتجة من تأثير رابع كلوريد الكربون على الكبد من إحداث تليف به- ودراسة التأثير المعاكس ل لـ- كرنتين والميلاتونين فى مقاومة تأثير رابع كلوريد الكربون .
وقد استخدم فى هذه البحث 72 من ذكور الجرذان البيضاء ووزعت كالآتي:-
المجموعة الأولى وهى المجموعة الضابطة وقد تم معالجتها بزيت البرافين مرتين فى الأسبوع لمدة ثمانية أسابيع.
المجموع الثانية وهى مكونة من 36 جرذا تم حقنهم برابع كلوريد الكربون فى الغشاء البريتونى بجرعة مقدارها 15. و ملجم / جرذ مرتين فى الأسبوع لمدة ثمانية أسابيع لإصابة الفئران بتليف الكبد- تم اخذ 12 منهم وذبحهم.
المجموعة الثالثة: تم اخذ 12 جرذا أخرى من التى أحدث بها تليف فى الكبد وتم معالجتها ب ل- كرنتين لمدة 4 أسابيع أخرى بجرعة مقدارها 50 ملجم/كجم.
المجموعة الرابعة: تم اخذ 12 جرذا بعد معالجتهم برابع كلوريد الكربون أي مصابين بتليف الكبد وتم معالجتهم بالميلاتونين بجرعة مقدارها 10 ملجم/كجم لمدة 4 أسابيع.
المجموعة الخامسة : تم معالجتها ب ل- كرنتين بجرعة مقدارها 50 ملجم/كجم عن طريق الفم لمدة 4 أسابيع .
المجموعة السادسة: وتم معالجتها بالميلاتونين بجرعة مقدارها 10 ملج/كجم لمدة 4 أسابيع.
تم إعداد قطاعات شمعية صبغت بالهيماتوكسلين والايوسين وتم إعدادها للفحص الهستولوجى. وتم إعداد صبغة ماسون الثلاثية لقياس المناطق المتليفة من الكبد باستخدام جهاز تحليل الصورة بالكمبيوتر. وتم عمل صبغات للفحص الهستوكيميائى وهى طريقة فولجن لفحص حمض ا لدى أكسى ريبونيو كليك وطريقة البير أيوديك –شف لعديدات التسكر المخاطية وطريقة البروموفينول الأزرق لتبيان كمية البر وتين باستخدام جهاز تحليل الصورة.
وقد أدت معالجة الفئران برابع كلوريد الكربون الى حدوث بعض التغيرات الهستولوجية فى الكبد مثل تكاثر الأنسجة الليفية مع ظهور بعض الفصيفصات الكاذبة مما يؤدى إلى فقدان الشكل المميز لخلايا الكبد وهذه الأنسجة الليفية تعمل كفاصل ما بين كل جزء و آخر و قد أظهر الفحص بجهاز تحليل الصورة زيادة معنوية فى المنطقة المتليفة .
اظهر الفحص الهستوكيميائى عن طريق جهاز تحليل الصورة نقصا فى محتوى الحامض النووى ا لدأكسى ريبونيو كليك ووجود نسبة حوالى 3% من الخلايا السرطانية وكذلك نقص فى كمية عديدات التسكر المخاطية وفى كمية البروتين.

أدي معالجة الجرذان بـ ل كرنيتين و الميلاتونين إلى تقليل نسبة الآثار السامة لرابع كلوريد الكربون على كبد الجرذان البيضاء وكان ذلك اكثر وضوحا فى حالـة الميلاتـونين عنـه فـى حالة ل كرنيتين.